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(54) Title: FUNGUS RESISTANT TRANSGENIC PLANTS

(57) Abstract

A method of conferring fungal resistance on plants is described. In the method, a DNA construct which encodes a *Stylosanthes humilis* peroxidase isogene or the like is introduced into cells of the plant. The construct is stably incorporated into the plant genome.

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FUNGUS RESISTANT TRANSGENIC PLANTS

TECHNICAL FIELD

This invention relates to transgenic plants with enhanced resistance to fungal pathogens. According to the invention, a transgenic plant is prepared by the transfer of a DNA sequence into the plant, which DNA sequence encodes a particular peroxidase enzyme. More specifically, the invention relates to the use of a *Stylosanthes humilis* peroxidase isogene in transgenic plants for the purpose of enhanced disease protection.

BACKGROUND ART

Diseases of crop plants have a considerable impact on the agricultural industries causing millions of tons of crop losses every year. Consequently, breeding resistant plant varieties using genes from compatible species has been the major objective of many plant breeding programs. With the advent of recombinant DNA techniques it has become possible to transfer genes between incompatible plant species to improve characteristics of a desired plant. One approach to protecting plants against microbes is to engineer the over-expression of plant genes that play a role in plant defence.

Plants can resist attack by a pathogen via a of complex network of defence mechanisms (Dixon and Harrison, *Adv. Genet.* 28:165-234 [1990]). Plants defence systems may include formation of physical barriers (cutin, lignin, callose), the expression of low molecular weight antibiotic compounds (phytoalexins) and anti-fungal proteins. Ectopic over-expression of anti-fungal proteins such as chitinases and β-1,3-glucanases and other plant proteins such as ribosome inactivating proteins have shown to mediate increased protection against phytopathogens (Broglie *et al.*, *Science* 254:1194-1197 [1991]; Jach *et al.*, *Plant J.* 8:97-109 [1995]; Liu *et al.*, *Bio/Technology* 13:686-691 [1994]; Logeman *et al.*, *Bio/Technology* 10:305-308 [1992]). One class of defence-related enzymes frequently hypothesised to have a role in defence are the peroxidases but to our knowledge the genes encoding these enzymes have not been successfully used in transgenic plants to engineer disease resistance.

Peroxidases (E.C. 1.11.1.7, donor:hydrogen-peroxidase oxidoreductase) have been implicated in a number of physiological functions that may be important in plant-pathogen interactions. These include lignification (Walter, M.H. in "Genes Involved in Plant Defense" T. Boller and F. Meins, eds. Springer-Verlag, Wien, New York pp. 327-352 [1992]), cross-linking of cell wall components (Bradley *et al.*, *Cell* 70:21-30 [1992]), wound healing (Sherf *et al.*, *Plant Physiol.* 101:201-208 [1993]) and auxin oxidation (Grambow and Langenbeck-Schwich, *Planta* 157:131-137 [1983]). Some isoforms of peroxidases are also shown to be inducible by pathogens (Svalhein and Robertson, *Physiol. Plant Physiol.* 78:261-267 [1990]; Kerby and Sommerville, *Plant Physiol.* 100:397-402 [1992]) and by wounding (Lagrimini and Rothstein, *Plant Physiol.* 84:438-442 [1987]). In addition there is substantial correlative evidence suggesting that peroxidase has a role in disease resistance.

Association of some peroxidase isoforms with systemic acquired resistance and hypersensitive responses have been demonstrated (Ye *et al.*, *Physiol. Mol. Plant Pathol.* 36:523-531 [1990]; Irwing and Kuc, *Physiol. Mol. Plant Pathol.* 37:355-366 [1990]). A high level of constitutive peroxidase expression (as well as other defence-related enzymes) in a hybrid between *Nicotiana glutinosa* x *N. debneyi* was also found to be associated with resistance to a number of tobacco pathogens including *Phytophthora parasitica var. nicotiana* (Goy *et al.*, *Physiol. Mol. Plant Pathol.* 41:11-21 [1992]). High levels of peroxidase activity has been used as a marker for resistance to downy mildew in muskmelon (Reuveni *et al.*, *Phytopthol.* 4 82:749-753 [1992]). Peroxidase enzymes can generate toxic radicals which are inhibitory to the growth of fungal pathogens *in vitro* (Peng and Kuc, *Phytopathol.* 82: 696-699 [1992]). In animal systems, peroxidases have also been implicated in defense against microbial and protozoan pathogens (Smith *et al.*, *Science* 268: 284-286 [1995] and Odell and Segal, *Biochim. Biophys. Acta* 971:266-274 [1988]).

Several investigators have cloned and studied the regulation and function of particular peroxidase isogenes from various species (Lagrimini *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7542-7546 [1983]; Buffard *et al.*, *Proc. Natl. Acad. Sci. USA* 87:8874-8878 [1990]; Roberts and Kolattukudy, *Mol. Gen. Genet.* 217:223-232 [1989]). Expression of particular peroxidase isogenes during the infection process has also been demonstrated. For instance, in tomato at least three different peroxidase genes are induced by infection (Mohan and Kolattukudy, *Plant Physiol* 92:276-280 [1990]; Sherf *et al.* [*Supra*]; Vera *et al.*, *Mol. Plant. Microbe Interact.* 6:790-794 [1993]). *Erysiphe graminis f. sp. hordei* infection in barley differentially induces two distinct peroxidase isogenes (Thordal-Christensen *et al.*, *Physiol. Mol. Plant Pathol.* 40:395-409 [1992]).

Previous work at the Cooperative Research Centre for Tropical Plant Pathology has shown that infection of the tropical forage legume *Stylosanthes humilis* by *Colletotrichum gloeosporioides* also induces peroxidase activity. Several peroxidase cDNA clones were isolated from *S. humilis* and a peroxidase isogene corresponding to Sphx6 was found to be strongly induced by the pathogen 4 hours after inoculation (Harrison *et al.*, *Mol. Plant Mic. Inter.* 8:398-406 [1995]). This time point precedes the primary penetration event demonstrating that early recognition and signalling process are involved in peroxidase gene expression during fungal infection.

It is evident from these studies that only some of the isoforms of peroxidases may be involved in plant pathogen interaction. Constitutive expression of such peroxidase isogenes in transgenic plants may lead to a disease resistant phenotype.

Several investigators have constitutively expressed peroxidase isogenes in transgenic plants (Sherf and Kolattukudy, *Plant J.* 3:829-833 [1993]; Lagrimini *et al.*, *J. Amer. Soc. Hort. Sci.* 117:1012-1016 [1992]; Lagrimini, *Plant Physiol.* 96:577-583 [1991]). However, there has been no report regarding disease resistant phenotypes of such plants expressing high levels of peroxidases.

Australia Patent Application No AU-B-52183/90 discloses a cucumber basic peroxidase cDNA

clone and chimaeric genes constructed using this clone for possible expression in transgenic plants for enhanced disease resistant phenotype. However, the peroxidase gene described in this document does not have any close overall homology to the Shpx6 peroxidase gene. Additionally, inoculation data is not given in AU-B-52183/90 so there is no evidence of the successful application of cucumber basic peroxidase in genetically engineering disease resistance in transgenic plants.

SUMMARY OF THE INVENTION

One of the objects of the present invention is to provide a method of genetically engineering plants so as to provide plants having an enhanced disease resistance phenotype with respect to wild type plants.

Another object of the present invention is to provide transgenic plants capable of constitutive expression of a peroxidase activity thereby providing an enhanced disease resistance phenotype with respect to the wild type plants.

According to a first embodiment of the invention, there is provided a method of engineering a plant to fungal resistance, the method comprising introducing into cells of the plant a DNA construct comprising:

(a) a promoter constitutively operative in the plant cell; and

(b) a DNA sequence encoding a peroxidase isozyme operatively linked to said promoter, wherein said DNA sequence is selected from:

(i) Shpx6 herein defined;

(ii) a sequence which hybridises to Shpx6 under stringent conditions and which encodes a protein having peroxidase activity;

(iii) a fragment of a DNA sequence according to (i) or (ii), which fragment encodes a protein having essentially the same activity as the peroxidase isozyme encoded by Shpx6.

According to a second embodiment of the invention, there is provided a plant cell harbouring a DNA construct comprising:

(a) a promoter constitutively operative in the plant cell; and

(b) a DNA sequence encoding a peroxidase isozyme operatively linked to said promoter, wherein said DNA sequence is selected from:

(i) Shpx6 herein defined;

(ii) a sequence which hybridises to Shpx6 under stringent conditions and which encodes a protein having peroxidase activity;

(iii) a fragment of a DNA sequence according to (i) or (ii), which fragment encodes a protein having essentially the same activity as the peroxidase isozyme encoded by Shpx6.

According to a third embodiment of the invention, there is provided a plant comprising cells

according to the second embodiment.

According to a fourth embodiment of the invention there is provided reproductive material, vegetative material or other regenerable tissue of the plant according to the third embodiment.

Other aspects of the invention will become apparent from a reading of the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the relevant portion of the binary vector (pGA643) containing a promoter-Sphx6-terminator construct.

Figure 2 shows the level of total leaf peroxidase activity in transgenic T_1 and T_2 tobacco families and in an untransformed control family.

Figure 3 shows the level of total leaf peroxidase activity in transgenic T_1 and T_2 canola families and in an untransformed control family.

Figure 4 shows inoculation data of transgenic and control tobacco families with *Phythopthora parasitica* cv *nicotiana*.

Figure 5 shows inoculation data of transgenic and control canola families (T_1, T_2 and T_3) with *Leptosphaeria maculans*.

Figure 6 shows glasshouse inoculations of adult plants of transgenic (T_1) and control canola families with *Leptosphaeria maculans*.

Figure 7 shows inoculation data of transgenic (T_1 and T_2) and control canola families with *Sclerotinia sclerotorium*.

BEST MODE AND OTHER MODES OF PERFORMING THE INVENTION

The following abbreviations are used hereafter:

PCR	polymerase chain reaction
BAP	6-benzylaminopurine
MS	Murashige-Skoog medium (Murashige and Skoog, *Physiologea Plantarum* 15:473-497 [1962], the entire contents of which are incorporated herein by cross-reference).
NAA	napthalene acetic acid

The present invention describes a process for the production of transgenic plants which have enhanced disease resistance. In this process, a chimaeric gene is constructed and transferred to plants using any of the well established methods of plant transformation which include *Agrobacterium* mediated transformation (Horsch *et al.*, *Science* 227:1229-1231 [1985]), electroporation into protoplasts (Fromm *et al.*, *Nature* 319:791-793 [1986]) and biolistic bombardment with DNA coated tungsten or gold particles (Klein *et al.*, *Proc. Natl. Acad. Sci. USA* 85:8502-8505 [1988]).

Transgenic plant cells including the DNA construct of the invention can be propagated using conditions appropriate to the particular plant. Similarly, whole plants, or propagating material of the

plant, can be prepared from the initial transgenic cells using known methods and conditions.

Chimaeric genes according to the invention have as a basis the peroxidase isogene which can be isolated from the tropical forage legume *Stylosanthes humilis*. This isogene has been designated Sphx6 and is described in Harrison *et al.*, *Mol. Plant-Microbe Interact.* 8:398-406 (1995), the entire contents of which is incorporated herein by cross-reference.

The chimaeric gene constructs of the invention comprise:

1) a DNA sequence encoding the Shpx6 peroxidase (Genbank Accession # L36110; Harrison *et al.*, *supra*; SEQ ID NO:1 herein) or a sequence encoding a peroxidase having essentially the same characteristics as the Shpx6 peroxidase;

2) a suitable promoter with or without other regulatory elements for constitutive or inducible expression in plants of the peroxidase encoded by (1); and optionally,

3) a suitable sequence for termination of transcription in plants.

As indicated above and in the description of the first and second embodiments, chimaeric genes according to the invention comprise not only the Shpx6 peroxidase but also allelic variants and homologues of Shpx6. The homologue can be an alternative *S. humilis* gene or a gene of another plant species. The chimaeric genes can further include DNA sequences which hybridise with the Shpx6 peroxidase sequence under stringent conditions. Such stringent conditions can be defined as follows:

Wash solution	0.1xSSPE/0.1% SDS
Wash temperature	65°C
Number of washes	two

(1xSSPE is a solution consisting of 180 mM NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA, and which has a pH of 7.4).

DNA sequences for inclusion in constructs according to the invention can be prepared or isolated using any of the methods known to those of skill in the art. Such methods are described in Sambrook *et al.*, *Molecular Cloning: a Laboratory Manual*, 2nd Ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbour NY (1989) and Ausebel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., USA (1987-1995), the entire contents of which are incorporated herein by cross-reference. For example, an Shpx6 homologue or allelic variant can be isolated from a genomic or cDNA library using hybridisation probes derived from the Sphx6 sequence. The Sphx6 sequence can also be used to derive oligonucleotide primers which can be used to amplify desired gene sequences by PCR. Harrison *et al.* (*supra*) describe a method of isolating Shpx6 from *S. humilis* genomic DNA.

With reference to item (ii) above, the promoter can be selected to ensure strong constitutive expression of the peroxidase protein in most or all plant cells, it can be a promoter which ensures expression in specific tissues or cells that are susceptible to fungal infestation, and it can also be a

promoter which ensures strong induction of expression during the infection process. Examples of other regulatory sequences which can be included in constructs are enhancers, untranslated regions of some transcripts and intron sequences from eukaryotic genes which can be used in combination with the suitable promoter. It will be appreciated by those of skill in the art that a promoter is not essential and the peroxidase encoded by the DNA sequence can be stably expressed in plant cells without any promoter present in the construct provided that insertion of the DNA sequence into the genome is in such a position that the sequence is operatively linked to a native plant promoter or similar regulatory sequences.

Regarding item (iii) above, transcription terminators operative in plant cells are well known in the art and are described, for example, in Ingelbrecht *et al., The Plant Cell* 1:671-680 (1989), the entire contents of which is incorporated herein by cross-reference. A preferred terminator is the Sphx6 terminator or the terminator of a homologue or allelic variant. However, depending on the site of insertion of the construct into a plant genome, a terminator may not be required and terminators naturally present in the genome of the transformed plant may be utilised.

The DNA constructs of the invention can be introduced into both monocotyledonous and dicotyledonous plants. The plant is typically from a family of plants of agricultural importance such as cereals, legumes, oilseed plants, sugar and fibre plants. However, plants that are not of agricultural importance can be transformed with the subject DNA constructs so that they exhibit a greater degree of resistance to fungal infestation. Specific examples of plants which can be genetically modified with DNA constructs according to the invention are maize, banana, peanut, field peas, sunflower, tomato, canola, tobacco, wheat, barley, oats, potato, soybeans, cotton, carnations, and sorghum.

Plant cells can be transformed with DNA constructs of the invention according to a variety of known methods (*Agrobacterium*, Ti plasmids, electroporation, micro-injections, micro-projectile gun, and the like) as has been briefly discussed above. Two such suitable methods will now be described.

Firstly, the DNA construct can be ligated into a binary vector carrying: i) left and right border sequences that flank the T-DNA of the *Agrobacterium tumefaciens* Ti plasmid; ii) a suitable selectable marker gene for the selection of antibiotic resistant plant cells; iii) origins of replication that function in either *A. tumefaciens* or *Escherichia coli*; and, iv) an antibiotic resistance gene that allows selection of plasmid-carrying cells of *A. tumefaciens* and *E. coli*. This binary vector carrying the chimaeric DNA construct can be introduced by either electroporation or triparental mating into *A. tumefaciens* strains carrying disarmed Ti plasmids such as strains LBA4404, GV3101, and AGL1 or into *A. rhizogenes* strains such as R4 or NCCP1885. These *Agrobacterium* strains can then be co-cultivated with suitable plant explants or intact plant tissue and the transformed plant cells and/or regenerants selected by using antibiotic resistance.

A second method of gene transfer to plants can be achieved by direct insertion of the gene in

target plant cells. For example, the DNA construct can be co-precipitated onto gold or tungsten particles along with a plasmid encoding a chimaeric gene for antibiotic resistance in plants. The tungsten particles can be accelerated using a fast flow of helium gas and the particles allowed to bombard a suitable plant tissue. This can be an embryogenic cell culture, a plant explant, a callus tissue or cell suspension or an intact meristem. Plants can be recovered using the antibiotic resistance gene for selection and antibodies used to detect plant cells expressing the peroxidase protein.

As described above in the fourth embodiment, the invention provides reproductive material, vegetative material, or other regenerable tissue of a plant which includes a DNA construct according to the invention. Seeds and pollen are included within the ambit of reproductive material and stem segments or cuttings within the ambit of vegetative material.

The invention will now be illustrated by the following non-limiting examples.

General Methods

Manipulation of DNA and RNA was carried out using known methods such as those described by Sambrook *et al.* (*Molecular Cloning: a Laboratory Manual*, 2nd Ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbour NY [1989]).

Reagents and other material were obtained from commercial sources or as otherwise indicated.

EXAMPLE 1

Construction of a chimaeric gene

In this example, we describe the construction of a chimaeric gene comprising a constitutive promoter and the coding region of Shpx6. The coding region of the Shpx6 cDNA (nucleotides 42 to 1001 of the SEQ ID NO:1 sequence - see SEQ ID NO:2 for the amino acid sequence) was amplified from plasmid pBluescript II SK$^+$ (Strategene) by the polymerase chain reaction (PCR) using the following oligonucleotide primers:

Primer 1 5' GGCTCTAGAAGTCGACATGGTTCG 3'

Primer 2 5' AACAGCTATGACCATG 3'.

The Primer 1 and 2 sequences were selected either wholly or at least partially from plasmid sequence either side of the Shpx6 insert. PCR primers were designed to incorporate restriction endonuclease sites to facilitate manipulation of the construct in general purpose cloning (pBluescript) and binary vectors for *Agrobacterium* based plant transformation.

PCR products were digested with *Xba*I and ligated into pBluescript cut with the same enzyme. Insertion of the Shpx6 cDNA was verified by DNA sequencing of the insert. DNA sequencing was performed on denatured double stranded DNA templates using automated methods on an Applied Biosystems (ABI) 373A instrument with the ABI PRISM Dye Deoxy Terminator Cycle Sequencing Kit. The sequence was verified on both strands with overlap. Oligonucleotide primers used for DNA sequencing were synthesised on a Beckman Oligo 1000 DNA synthesiser. Shpx6 insert was

later separated from the pBluescript DNA with *Xba*I and cloned into a binary vector - pGA643 (An *et al., EMBO J.* 4:227-288 [1985]). This created a transcriptional fusion between the constitutive expression promoter and the Shpx6 cDNA. Figure 1 schematically illustrates this fusion construct.

The binary vector carrying the chimaeric gene construct was then used to transform tobacco and canola using an *Agrobacterium tumefaciens* mediated transformation system.

EXAMPLE 2

Preparation of transgenic plant cells

Agrobacterium tumefaciens was transformed with the vector carrying the chimaeric construct using electroporation (Nagel *et al., FEMS Microbiol. Let.* 67:325-328 [1990]). Both tobacco and canola were transformed using *A. tumefaciens* strain LBA4404 (GibcoBRL). Tobacco (*N. tabacum*) transformation was carried out essentially according to Horsch *et al.* (*Science* 227:1229-1231 [1985]) using leaf discs and 100 mg/L kanamycin as selective agent. For canola transformation, seeds of a double haploid canola line (141-227) derived from cv. Westar produced in the Crop Science Department of the University of Guelph and Ontario Ministry of Agriculture were obtained from Dr. W.D. Bewersdorf (Crop Science Department of the University of Guelph, Ontario, Canada). Seeds from this line were surface sterilised and germinated on MS salts (Murashige and Skoog, *supra*) complemented with 3% sucrose and 0.8% agar under a regime of 16 h light and 8 h dark at 24 °C. Hypocotyl segments (5-10 mm in length) were taken from 5 to 6 day-old sterile seedlings and preincubated for a day on callus-inducing medium including MS salts and vitamins, 3% sucrose, 1 mg/L 2,4-D and 0.8% Difco Bacto-agar. *Agrobacterium tumefaciens* harbouring peroxidase gene constructs was grown overnight in YEP medium (An *et al., Plant Physiology* 81:301-305 [1988]) with selective antibiotics. Before cocultivation, the absorbance at A_{660} of the bacterial solution was determined and the number of bacteria was adjusted to $1x10^8$ per mL ($A_{660}=0.03$) in liquid callus inducing medium. Hypocotyl segments were incubated in bacterial solution with gentle shaking for 5 min, blotted on sterile filter papers placed on callus inducing medium for 2-3 days. After cocultivation, the segments were washed twice in liquid MS medium, blotted briefly on filter paper and placed on MS medium solidified with Phytagar (Difco) or Phytagel (Sigma) and containing 150 mg/L timentin (Ticarcillin). After 5-7 days incubation on this medium, segments were transferred on shoot regeneration medium containing 3 mg/L BAP, 1 mg/L zeatin, 5 mg/L $AgNO_3$, 25 mg/L kanamycin and 150 mg/L timentin. Plates were sealed with Micropore tape (3M Health Care, MN, USA). The initial plating densities were 40-50 explants per plate. This was reduced to 20-25 per plate in subsequent subcultures. Hypocotyl segments were subcultured onto fresh medium without $AgNO_3$ every two weeks. Differentiated shoots were transferred to jars. Elongation and root formation were established in a hormone-free medium containing half strength MS and sucrose, 25 mg/L kanamycin and 100 mg/L timentin. The transgenic status of the shoots was assessed by placing leaf discs on a medium containing 4 mg/L BAP, 0.5 mg/L NAA and 25-50 mg/L kanamycin

for four weeks. Rooted transgenic shoots were transferred to soil and kept under a dome for a few weeks in controlled environment rooms before exposing the shoots to normal conditions.

EXAMPLE 3

Peroxidase assays for the analysis of transgenic plant tissue expressing Shpx6

Freshly harvested leaves from transgenic (T_0, T_1, T_2 and T_3) *N. tabaccum* cv. Xanthi and *B. napus* cv. Westar (141-227) were frozen in liquid N_2 for storage and subsequently homogenised at 4°C in a microcentrifuge tube with a custom made tight-fitting stainless steel grinder attached to an electric drill using 3 volumes per unit fresh weight of buffer (10 mM sodium phosphate, 1% sodium metabisulphite, pH 6.0). Homogenates were centrifuged at 14,000 rpm in a refrigerated microfuge at 4°C for 30 minutes and aliquots of supernatant frozen at -70°C. Peroxidase assays were carried out according to Rathmell and Sequeira (*Plant Physiol* 53:317-318 [1974]). Reactions contained 0.28 % guaiacol and 0.3 % H_2O_2 in 50 mM sodium phosphate buffer (pH 6.0). The reaction rate was monitored at 470 nm. Reaction rates were linear and proportional to the enzyme concentration added. Total protein was determined using the Bio-Rad protein assay adapted for microtitre plates.

Figures 2 and 3 show the total leaf peroxidase activities of the T_1 and T_2 transgenic tobacco and canola families, respectively. Depending on the transgenic family, constitutive over-expression of Sphx6 resulted with 2-3 fold increases in the total leaf peroxidase activity over untransformed control plants. In these figures, peroxidase activity in 10-20 plants from each transgenic and control family was measured and values for t were calculated in pairwise comparison of the transgenic families with the control family. Standard deviations are indicated as arrows. Families with different denoted letters show significant differences at $P < 0.05$.

EXAMPLE 4

Development of transgenic T1 seed lines

Genotype designations for transgenic plants used herein are in accordance with the following convention: the initial plant resulting from a transformation event and having grown from tissue culture is designated a T_0 plant. Plants resulting from self pollination of the natural flowers of the T_0 plant are designated T_1.

Transgenic plants (T_0) were grown to maturity. Flowers were allowed to self-pollinate and seed pods collected after normal desiccation. Seeds from each individual plant were collected and stored separately. Each seed lot was tested by genetic segregation analysis to determine the number of Mendelian loci bearing the kanamycin resistant trait. Seeds collected from each T_0 plant were germinated on MS medium containing 400 mg/L kanamycin. The ratio of normal green (kan-r) versus bleached (kan-s) cotyledons was determined. Seedlings with green colour were transplanted to soil for further analyses.

To produce a further generation, seeds were collected from T_1 plants and the above process

repeated to produce T_2 plants. T_3 plants were similarly produced.

EXAMPLE 5

Evaluation of transgenic plant tissue expressing Shpx6 for disease resistance

In this example, we describe the response of transgenic plants capable of expressing Shpx6 peroxidase to challenge from a variety of fungal pathogens.

Eight-week old transgenic tobacco plants from T_1 and T_2 transgenic families were inoculated with the fungal pathogen *Phytophthora parasitica* var. *nicotiana* (black shank disease of tobacco) using the methods described by Robin and Guest (*NZ J. Crop Hort. Sci.* 22:159-166 [1994]). Ten plants were used for each family (transgenes and controls). Lesion lengths on the decapitated stems were measured daily for 8 days postinoculation. Values for t were calculated in pairwise comparison of the transgenic families with control untransformed plants.

The results of this experiment are presented in Figure 4 in which the filled bars represent T_1 families and the vertically hatched bars represent T_2 families. The error bars represent the standard deviation for each family. Families showing significant differences at $P<0.05$ with respect to the controls are denoted by different letters above the error bars. Analysis of inoculation data from this experiment showed that the transgenic families with higher peroxidase activity had significantly better protection with respect to wild type plants.

For *Leptosphaeria maculans* (blackleg disease of canola) inoculations, cotyledons from T_1, T_2 and T_3 canola seedlings were punctured and inoculated with the pycnidiospore suspension of 10^4 spores. Disease reaction, or index, was scored visually using a scale where "0" corresponded to complete resistance and "9" corresponded to complete susceptiblity to infection. Based on this scale, plants with an index of 0-3 were considered resistant, plants with an index of 4-6 moderately resistant, and plants with an index of 7-9 susceptible. Thirty plants or more were used for each transgenic family and untransformed control families. Duncan's Multiple Range Test were used to statistically compare transgenic families with the untransformed controls.

Data from these inoculation experiments are presented in Figure 5. In Figure 5, the horizontally hatched bars represent T_1 families, the vertically hatched bars represent T_2 families, and the filled bars represent T_3 families. Analysis of the data showed that some of the transgenic lines had significantly better protection against *Leptosphaeria* ($P<0.05$). To measure the response of adult plants to this pathogen, an inoculation experiment using 5-6 weeks old plants from T_1 families were also done in the glasshouse. In this experiment, plant survival rate for each family was calculated as the percentage of plants that reached maturity and set seed. Data from this inoculation experiment are presented in Figure 6. Analysis of data showed that transgenic lines which performed better in cotyledon inoculation tests displayed better survival rates.

For *Sclerotinia sclerotorium* inoculations, stems of 10 adult canola plants from each of T_1 and

T_2 families were inoculated by securing a barley grain colonised by the fungus on the stem. Lesion extension was measured daily. Duncan's Multiple Range Test were used to statistically compare transgenic families with untransformed control plants. Data from the inoculation experiment are presented in Figure 7 in which the filled bars represent T_1 families and the vertically hatched bars represent T_2 families. Experiments with T_1 families showed that some transgenic lines expressing Shpx6 had better protection against the fungus due to lower rates of lesion extension on their stems. However, next round inoculations done on T_2 families did not show any significant protection. Peroxidase assays done on a subset of plants sampled before the inoculations also showed some reduction in the total peroxidase activity of these plants. This suggested that stable expression of transgene (Shpx6) is necessary for consistent disease resistance responce of canola against *S. sclerotorium*.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: COOPERATIVE RESEARCH CENTRE FOR TROPICAL PLANT PATHOLOGY
(B) STREET: The University of Queensland
(C) CITY: St Lucia
(D) STATE: Queensland
(E) COUNTRY: Australia
(F) POSTAL CODE (ZIP): 4067

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(B) STREET: National Circuit
(C) CITY: Barton
(D) STATE: ACT
(E) COUNTRY: Australia
(F) POSTAL CODE (ZIP): 2600

(A) NAME: KAZAN, Kemal (US only)
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(C) CITY: St Lucia
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(F) POSTAL CODE (ZIP): 4067

(A) NAME: GOULTER, Kenneth C. (US only)
(B) STREET: 26 Emblem Street
(C) CITY: Jamboree Heights
(D) STATE: Queensland
(E) COUNTRY: Australia
(F) POSTAL CODE (ZIP): 4074

(A) NAME: MANNERS, John M. (US only)
(B) STREET: 28 Warmington Street
(C) CITY: Paddington
(D) STATE: Queensland
(E) COUNTRY: Australia
(F) POSTAL CODE (ZIP): 4064

(ii) TITLE OF INVENTION: Fungus Resistant Transgenic Plants

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1144 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Stylosanthes humilis
(B) STRAIN: Paterson
(F) TISSUE TYPE: stem

(vii) IMMEDIATE SOURCE:
(B) CLONE: Shpx6

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION:42..113

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:114..1001

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Harrison, S J
Curtis, M D
McIntyre, C L
Maclean, D J
Manners, J M
(B) TITLE: Differential expression of peroxidase isogenes during the early stages of infection of the tropical forage legume Stylosanthes humilis by Colletotrichum gloeosporioides
(C) JOURNAL: Mol. Plant Microb. Interact.

(D) VOLUME: 8
(E) ISSUE: 3
(F) PAGES: 398-406
(G) DATE: 1995
(K) RELEVANT RESIDUES IN SEQ ID NO: 1: FROM 1 TO 1144

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GCAAAGTTTG TTTGATAATA TTGGTGATGA GCCTTATAGG ATTAGGATCA GGTCAATTGT 120

CATCAAATTT TTATGCAACA ACATGTCCGA ATGCACTTTC AACGATTAGG TCAGGAGTGA 180

ACTCTGCTGT GAGCAAAGAA GCTCGCATGG GAGCTTCCCT TCTTCGCCTT CATTTCCATG 240

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GAGAAAAAAC AGCACGTCCT AATGCTAATT CAATTAGAGG TTTTGAAGTC ATAGACACCA 360

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TTGCTGCTAG AGATTCTGTT GTTGCTCTTG GTGGACCCAG TTGGACAGTG CAACTGGGAA 480

GAAGAGACTC AACTACAGCA AGTTTAAGCT TAGCTAACTC AGATTTGGCT GCTCCCACTT 540

TGGATCTCAG TGGTCTAATC TCTGCTTTCT CTAAGAAAGG TTTATCAACT AGTGAAATGG 600

TTGCCCTATC AGGAGGGCAT ACAATTGGGC AAGCAAGATG CACAAGCTTT AGAACAAGGA 660

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AACTCTTCAA TGGAGGATCC ACAGATTCTC AAGTGAATGG TTATGCCTCC AACCCTTCAA 900

GTTTCTGCTC TGATTTTGGC AATGCTATGA TTAAGATGGG TAACATTAGT CCACTCACTG 960

GATCCAGTGG CCAGATTAGG ACCAATTGCA GGAAGACCAA TTAGGATCAT ATGATAAAAT 1020

AATTAATAAT ATAGATAAAA AATATATATA TATATATAAT AATAATAATA ATTAAATAAA 1080

CCGAATATAG TTTCTAGCTT ATAACTTTTG TTTTATTTTT TAATGTTGAA GAAATTAAAA 1140

GGGT 1144

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 320 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Stylosanthes humilis
(B) STRAIN: Paterson

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Ser Leu Ile Gly Leu Gly Ser Gly Gln Leu Ser Ser Asn Phe Tyr Ala
            20                  25                  30

Thr Thr Cys Pro Asn Ala Leu Ser Thr Ile Arg Ser Gly Val Asn Ser
        35                  40                  45

Ala Val Ser Lys Glu Ala Arg Met Gly Ala Ser Leu Leu Arg Leu His
    50                  55                  60

Phe His Asp Cys Phe Val Gln Gly Cys Asp Ala Ser Val Leu Leu Asp
65                  70                  75                  80

Asp Thr Ser Asn Phe Thr Gly Glu Lys Thr Ala Arg Pro Asn Ala Asn
                85                  90                  95

Ser Ile Arg Gly Phe Glu Val Ile Asp Thr Ile Lys Ser Gln Val Glu
            100                 105                 110

Ser Leu Cys Pro Gly Val Val Ser Cys Ala Asp Ile Leu Ala Val Ala
        115                 120                 125

Ala Arg Asp Ser Val Val Ala Leu Gly Gly Pro Ser Trp Thr Val Gln
    130                 135                 140
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Leu Gly Arg Arg Asp Ser Thr Thr Ala Ser Leu Ser Leu Ala Asn Ser
145 150 155 160

Asp Leu Ala Ala Pro Thr Leu Asp Leu Ser Gly Leu Ile Ser Ala Phe
165 170 175

Ser Lys Lys Gly Leu Ser Thr Ser Glu Met Val Ala Leu Ser Gly Gly
180 185 190

His Thr Ile Gly Gln Ala Arg Cys Thr Ser Phe Arg Thr Arg Ile Tyr
195 200 205

Thr Glu Ser Asn Ile Asp Pro Asn Phe Ala Lys Ser Leu Gln Gly Asn
210 215 220

Cys Pro Asn Thr Thr Gly Asn Gly Asp Asn Asn Leu Ala Pro Ile Asp
225 230 235 240

Thr Thr Ser Pro Thr Arg Phe Asp Asn Gly Tyr Tyr Lys Asn Leu Leu
245 250 255

Val Lys Lys Gly Leu Phe His Ser Asp Gln Gln Leu Phe Asn Gly Gly
260 265 270

Ser Thr Asp Ser Gln Val Asn Gly Tyr Ala Ser Asn Pro Ser Ser Phe
275 280 285

Cys Ser Asp Phe Gly Asn Ala Met Ile Lys Met Gly Asn Ile Ser Pro
290 295 300

Leu Thr Gly Ser Ser Gly Gln Ile Arg Thr Asn Cys Arg Lys Thr Asn
305 310 315 320

CLAIMS

1. A method of engineering a plant to fungal resistance, the method comprising introducing into cells of the plant a DNA construct comprising:

(a) a promoter constitutively operative in the plant cell; and

(b) a DNA sequence encoding a peroxidase isozyme operatively linked to said promoter, wherein said DNA sequence is selected from:

(i) Shpx6 herein defined;

(ii) a sequence which hybridises to Shpx6 under stringent conditions and which encodes a protein having peroxidase activity;

(iii) a fragment of a DNA sequence according to (i) or (ii), which fragment encodes a protein having essentially the same activity as the peroxidase isozyme encoded by Shpx6.

2. The method according to claim 1, wherein said promoter is the 35S promoter of Cauliflower Mosaic Virus.

3. The method according to claim 1, wherein said DNA sequence comprises SEQ ID NO: 1.

4. The method according to claim 1, wherein said peroxidase isozyme has an amino acid sequence substantially corresponding to SEQ ID NO: 2.

5. The method according to claim 1, wherein said fungal resistance is to *Phytophthora parasitica*, *Leptosphaeria maculans*, or *Sclerotinia sclerotorium*.

6. A plant cell harbouring a DNA construct comprising:

(a) a promoter constitutively operative in the plant cell; and

(b) a DNA sequence encoding a peroxidase isozyme operatively linked to said promoter, wherein said DNA sequence is selected from:

(i) Shpx6 herein defined;

(ii) a sequence which hybridises to Shpx6 under stringent conditions and which encodes a protein having peroxidase activity;

(iii) a fragment of a DNA sequence according to (i) or (ii), which fragment encodes a protein having essentially the same activity as the peroxidase isozyme encoded by Shpx6.

7. The plant cell according to claim 6, wherein said promoter is the 35S promoter of Cauliflower Mosaic Virus.

8. The plant cell according to claim 6, wherein said DNA sequence comprises SEQ ID NO: 1.

9. The plant cell according to claim 6, wherein said peroxidase isozyme has an amino acid sequence substantially corresponding to SEQ ID NO: 2.

10. The plant cell according to claim 6, wherein said fungal resistance is to *Phytophthora parasitica*, *Leptosphaeria maculans*, or *Sclerotinia sclerotorium*.

11. The plant cell according to claim 6, wherein said DNA construct is incorporated into the

genome of said plant cell.

12. A plant comprising cells according to claim 6.

13. The plant according to claim 12 which is a monocot or a dicot.

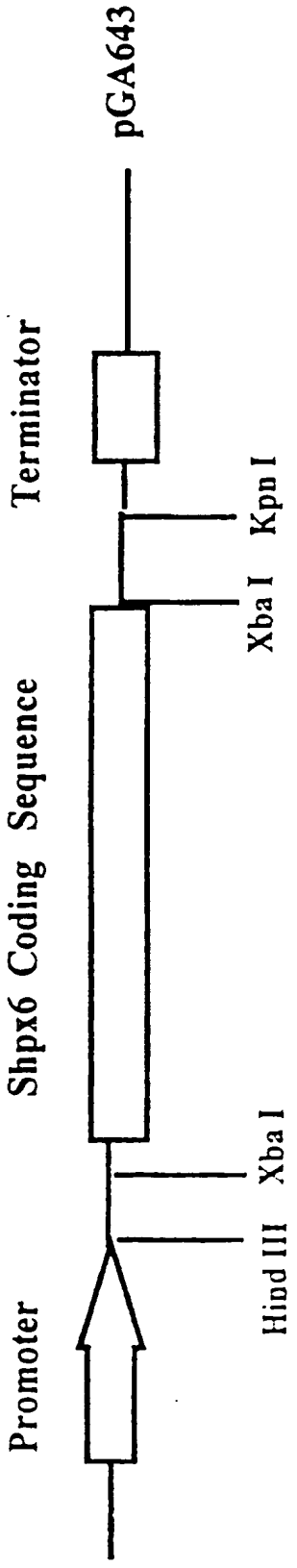
14. The plant according to claim 12 which is from a family of plants selected from cereals, legumes, oilseed plants, sugar or fibre plants.

15. The plant according to claim 12 which is selected from maize, banana, peanut, field peas, sunflower, tomato, canola, tobacco, wheat, barley, oats, potato, soybeans, cotton, carnations, or sorghum.

16. Material of the plant according to claim 12, which material is selected from reproductive material, vegetative material, or other regenerable material.

17. Material according to claim 16, wherein said reproductive material is seed or pollen.

18. Material according to claim 16, wherein said vegetative material is a stem segment or a cutting.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Kpn I
Xba I
Shpx6 Coding Sequence
Xba I
Hind III
Promoter

Fig. 1

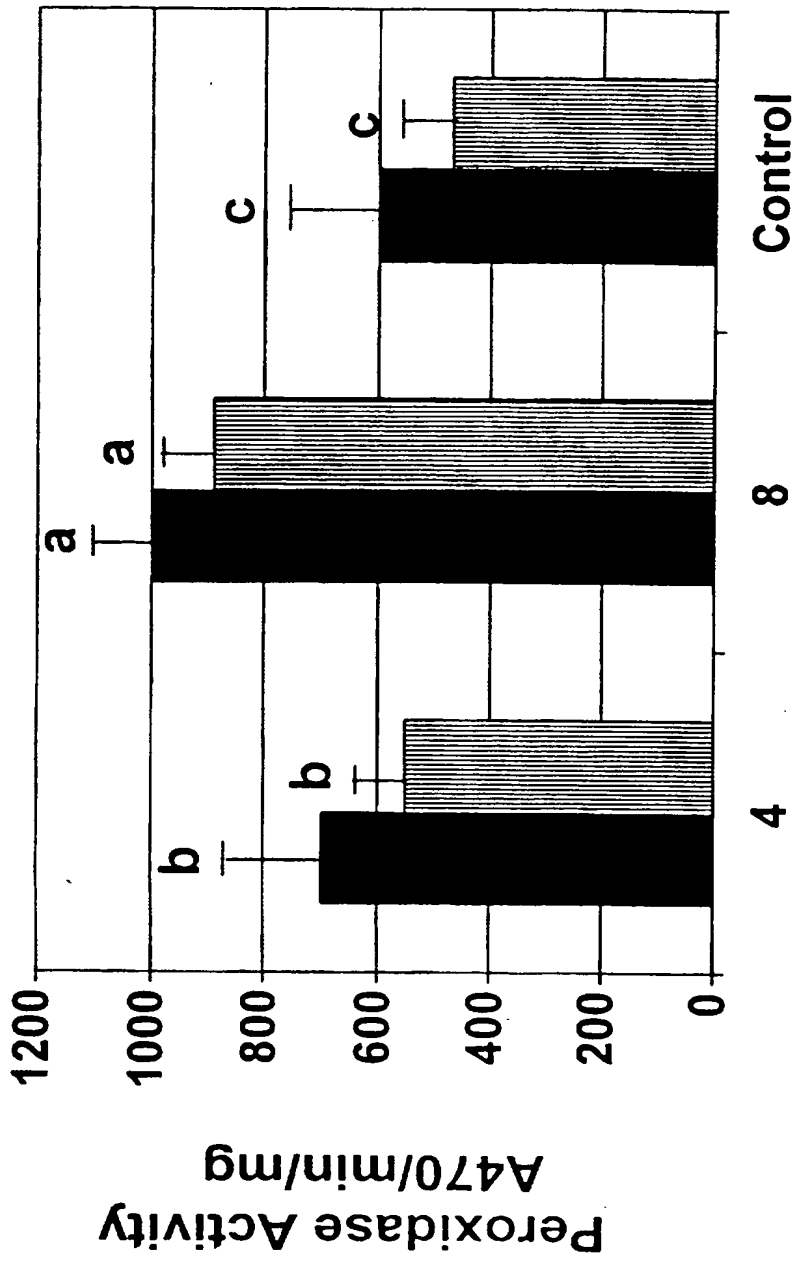

Peroxidase Activity
A470/min/mg
1200
1000
800
600
400
200
0
a
b
a
b
c
c
4
8
Control
Transgenic Family

Fig. 2

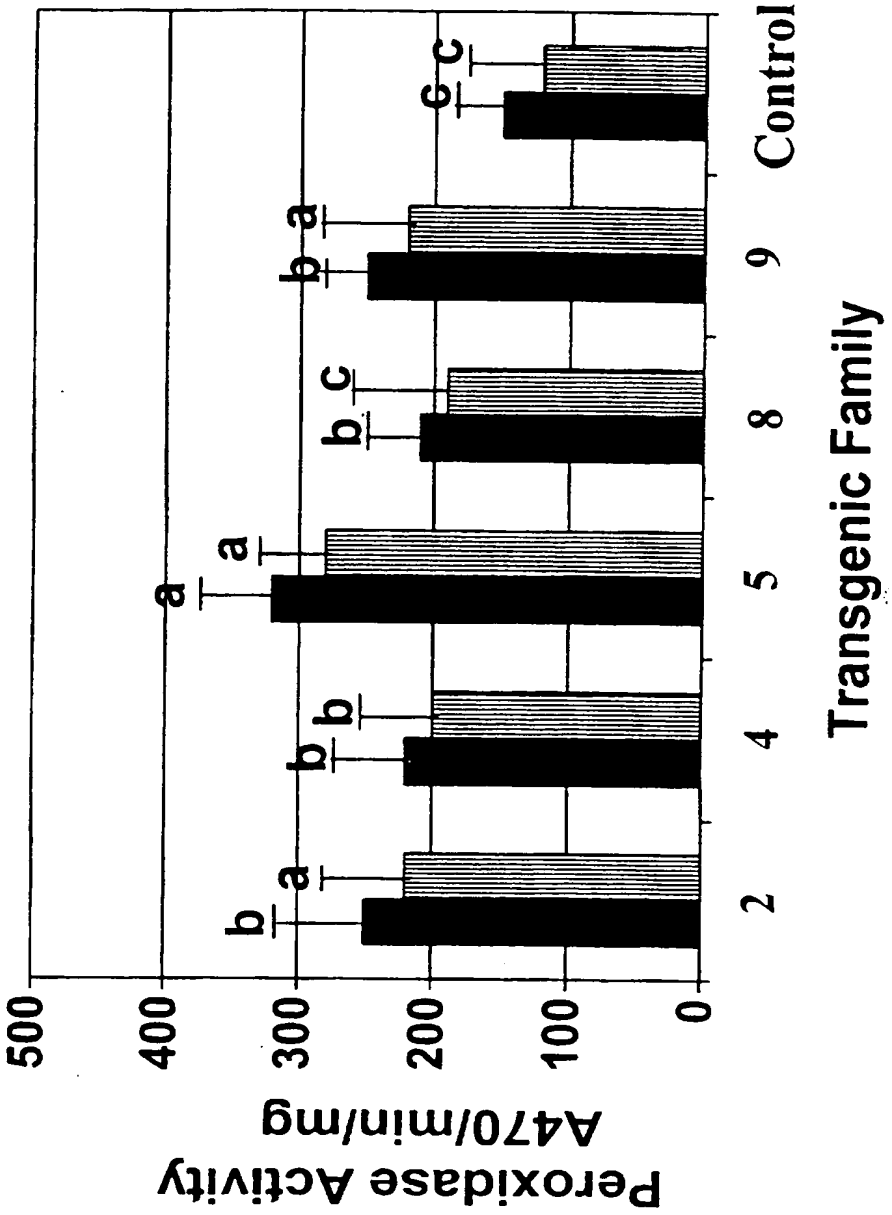
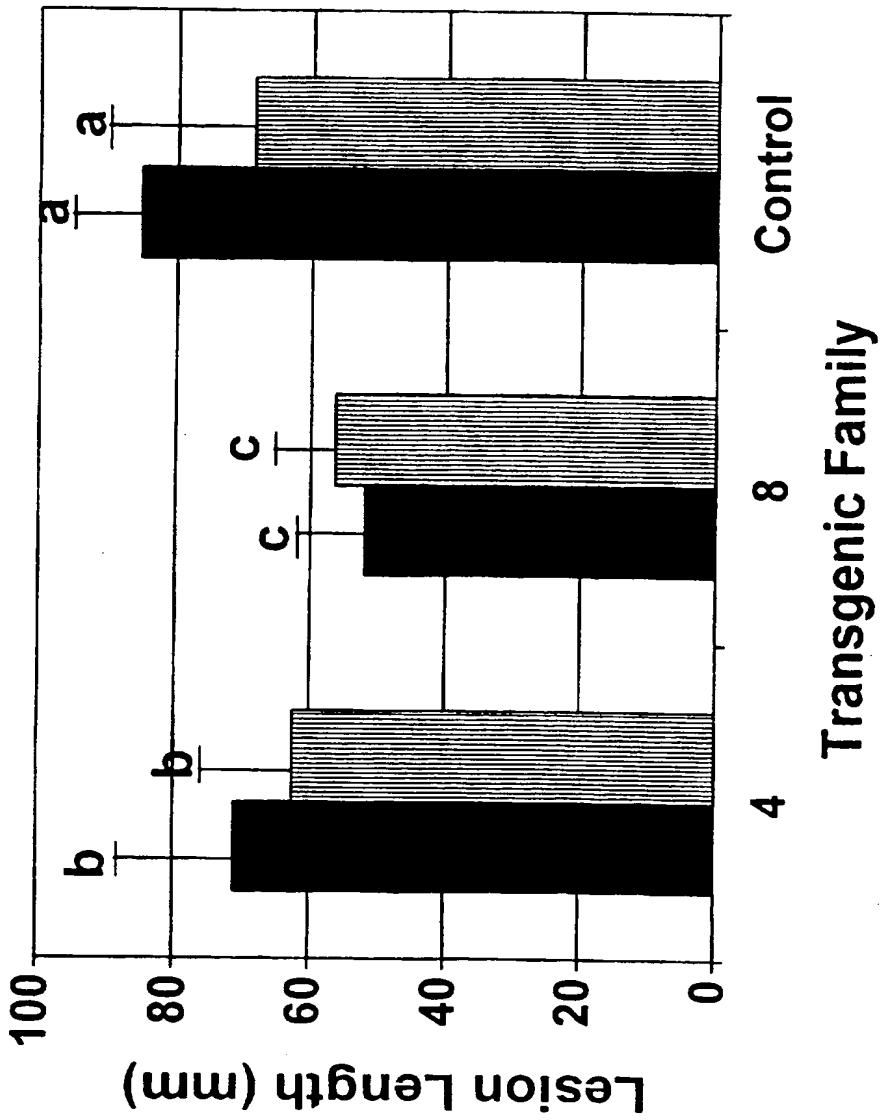


Fig. 3


Lesion Length (mm)
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80
60
40
20
0
a
a
c
c
b
b
4
8
Control
Transgenic Family

Fig. 4

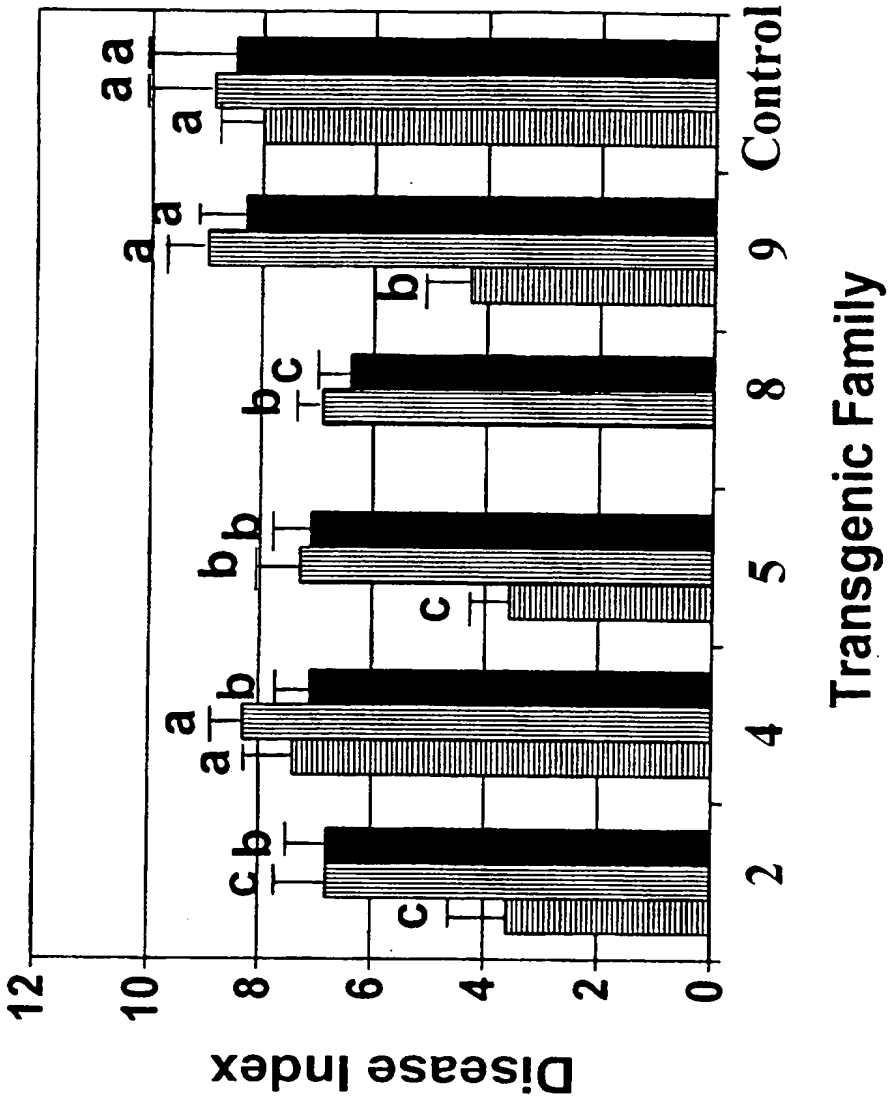
Disease Index
0
2
4
6
8
10
12
Transgenic Family
2
4
5
8
9
Control
a a
a
a a
b
b c
b b
c
a b
a
c b
c

Fig. 5

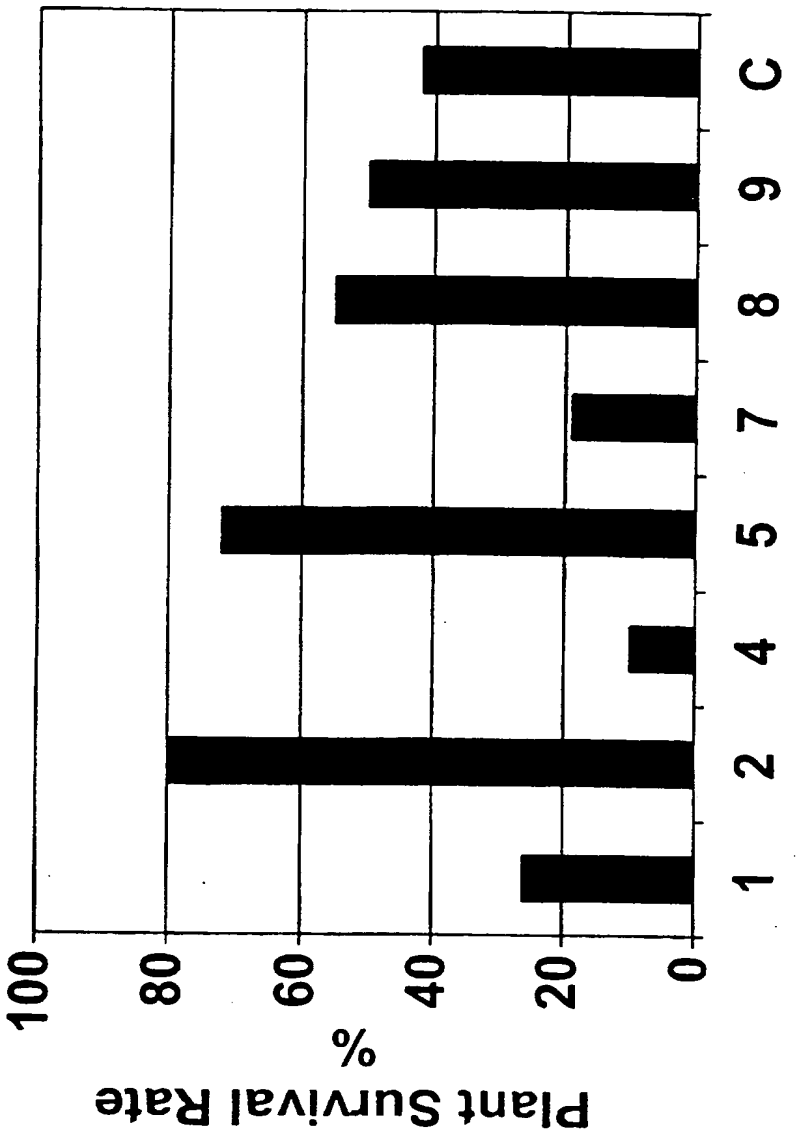
Plant Survival Rate
%
100
80
60
40
20
0
1
2
4
5
7
8
9
C
Transgenic Family

Fig. 6

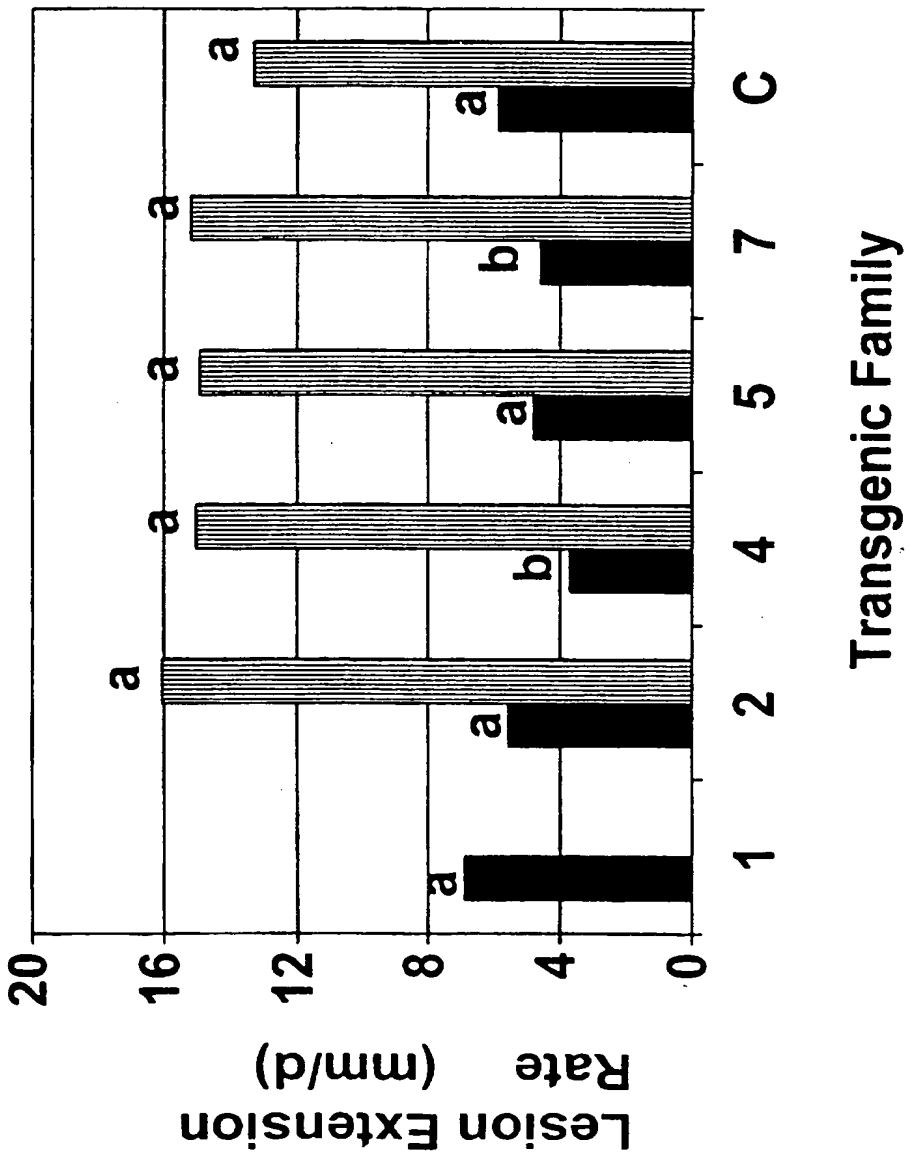

Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00253

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N-15/53 C12N-5/14 A01N-63/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
CHEMICAL ABSTRACTS: see below
WPAT: STYLOSANTHES and HUMILIS and PEROXIDE, HARRISON and PEROXIDE, MANNERS and PEROXIDE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
SEQ ID No. 2 searched; SwissProt, Genbank, EMBL, PIR
CAS online, STN (DGENE): KEARMGASLL RLHFHDCFVQ GCDASVLLDD TSNFTGEKTA GPNANSIRGF EVID (52-105 of SEQ ID No.2)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Molecular Plant-Microbe Interractions 8 (3), pages 398-406 (1995) Harrison, S.J. *et el* "Differential Expression of Peroxidase Isogenes During the Early Stages of Infection of the Tropical Forage Legume *Stylothanthes humilis* by *Cottetotrichum gloeosporioides*"	1-18
Y	Plant Physiol. 96, pages 577-83 (1991) Lagrimini L.M. "Wound Induced Deposition of Polyphenols in Transgenic Plants Overexpressing Peroxidase"	1-18
		1-18

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 MAY 1977	23 JUN 1997

Name and mailing address of the ISA/AU
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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00253

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AU-A-52183/90 (CIBA-GEIGY AG) 27 September 1990 see in particular pages 48-49	
A	Plant. Mol. Biol. 29, pages 647-62 (1995) Baga, M. *et al* "Molecular Cloning and Expression analysis of Peroxidase Genes From Wheat"	

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00253

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 97/00253

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member
AU-A	52183/90	CA 2012778
		EP 392225
		JP 3035783
		NZ 233053
		US 5614395
		ZA 9002250

END OF ANNEX